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Metalloprotease-Mediated Shedding of Enzymatically Active Mouse ecto-ADP-ribosyltransferase ART2.2 Upon T Cell Activation[1]

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Metalloprotease-Mediated Shedding of Enzymatically Active Mouse ecto-ADP-ribosyltransferase ART2.2 Upon T Cell Activation[1]

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T cells proteolytically shed the ectodomains of several cell surface proteins and, thereby, can alter their responsiveness and can release soluble intercellular regulators. ART2.2 is a GPI-anchored ecto-ADP-ribosyltransferase (ART) related to ADP-ribosylating bacterial toxins. ART2.2 is expressed exclusively by mature T cells. Here we show that ART2.2 is shed from the cell surface in enzymatically active form upon activation of T cells. Shedding of ART2.2 resembles that of L-selectin (CD62L) in dose response, kinetics of release, and sensitivity to the metalloprotease inhibitor Immunex Compound 3, suggesting that ART2.2, like CD62L, is cleaved by TNF-α-converting enzyme or by another metalloprotease. ART2.2 shed from activated T cells migrates slightly faster in SDS-PAGE analyses than does ART2.2 released upon cleavage of the GPI anchor. This indicates that shedding of ART2.2 is mediated by proteolytic cleavage close to its membrane anchor. Shed ART2.2 is enzymatically active and ADP-ribosylates several substrates in vitro. Thus, shedding of ART2.2 releases a potential intercellular regulator. Finally, using a new FACS assay for monitoring ADP-ribosylation of cell surface proteins, we demonstrate that shedding of ART2.2 correlates with a reduced sensitivity of T cell surface proteins to ADP-ribosylation. Our findings suggest that by shedding ART2.2 the activated T cell not only releases a potential intercellular regulator but also may alter its responsiveness to immune regulation by ART2.2-mediated ADP-ribosylation of cell surface proteins. ***The Journal of Immunology,*** **2000, 165: 4463–4469.**

Mammalian cells proteolytically shed the ectodomains of numerous cell surface proteins (1, 2). Modification of the cell surface in this way can alter the responsiveness of the cell and can release soluble intercellular regulators. Release of TNF-α provides a well-studied example of this mechanism. The membrane-anchored multidomain proteinase responsible for releasing TNF-α (TNF-α-converting enzyme (TACE)[3]) has been cloned and the three-dimensional structure of the catalytic domain cocrystalized with a hydroxamic acid-based inhibitor has been elucidated (3, 4). Recent evidence from TACE knockout mice indicates that TACE is also responsible for the release of L-selectin (CD62L) and TNF-αR from lymphocytes and monocytic cells, respectively (5). Shedding of TNF-α and CD62L is inhibited by specific inhibitors, such as Immunex Compound 3 (IC-3) (Immunex, Seattle, WA) (6).

We have previously reported the molecular cloning and biochemical characterization of a family of GPI-anchored mammalian cell surface mono-ADP-ribosyltransferases (ARTs) that are related in structure and function to bacterial ADP-ribosylating toxins (7, 8). ADP-ribosylation, like phosphorylation, is a posttranslational protein modification affecting important biological functions (9, 10). In the reaction, the ADP-ribose moiety of NAD^+ is transferred onto a specific amino acid residue in a target protein, while the nicotinamide moiety is released. In the context of the immune system, treatment of lymphocytes with ART inhibitors or the ART-substrate NAD^+ profoundly affects cellular functions including proliferation, cytotoxicity, homing, and TCR clustering (11–13). These effects have been associated with GPI-anchored ART, and LFA-1 and CD8 have been identified as key target proteins of ADP-ribosylation on the T cell surface (12, 14, 15). Evidence has also been reported for the release of ART activity into the cell supernatant upon activation of mouse allogeneic CTL and of chicken heterophils (16, 17). So far, neither the molecular identity of the cell surface ART(s) nor the mechanism for its (their) release have been elucidated.

We have recently developed a mAb, Nika102, specific for GPI-anchored mouse ecto-ART, ART2.2 (18). With Nika102 we demonstrated that expression of ART2.2 is developmentally regulated during postnatal ontogeny and that cell surface ART2.2 is detectable only on mature T cells. Moreover, we found that ART2.2 is down-modulated upon activation of T cells with anti-CD3 or PMA. Here we show that ART2.2 is shed from the cell surface in enzymatically active form by activated T cells. Dose response, kinetics of release, and sensitivity of shedding to the TACE inhibitor IC-3 strongly suggest that shedding is mediated by a pathway similar to the one that releases CD62L. Shed ART2.2 ADP-ribosylates a variety of targets in vitro and, thus, represents a potential soluble regulator of target proteins in vivo.

Materials and Methods

Mice

Mice were obtained from the Animal Resources Unit of The Jackson Laboratory (Bar Harbor, ME) and from the University Hospital (Hamburg, Germany). A stock of nonobese diabetic (NOD) mice doubly transgenic for

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[3] Abbreviations used in this paper: TACE, TNF-α converting enzyme; IC-3, Immunex Compound 3; ART, ADP-ribosyltransferase; sIg, surface Ig; PI-PLC, phosphatidylinositol-specific phospholipase C; NOD, nonobese diabetic.

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the rearranged Vα8 Vβ2 TCR of a diabetogenic CD8$^+$ T cell clone isolated from NOD/Lt islets (here designated NOD.AI4$\alpha\beta$Tg) were provided by Dr. David Serreze (The Jackson Laboratory) and are described elsewhere (19). Splenic T cells from these donors were activated in vitro by coculture with collagenase-isolated islets from NODLtSz.*scid*.RIPB7 mice expressing the costimulatory B7-1 membrane protein under control of the insulin promoter were also provided by Dr. Serreze and are described elsewhere (20).

Abs and immunofluorescence analysis

ART2.2-specific polyclonal rabbit antiserum K48 and mAbs Nika102 (rat IgG2a) and Nika109 (rat IgG1) were produced as described previously (18, 21). Mouse mAb 1G4 (IgG2a) specific for etheno-adenosine (22) was kindly provided by Dr. Regina Santella (Columbia University, New York, NY). Other mAbs used in this study for immunofluorescence staining and activation assays include anti-CD3e (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.72), anti-CD62L (Mel-14), and anti-CD38 (90). Biotin, PE, and FITC conjugates were purchased from PharMingen (San Diego, CA). GK1.5 (anti-CD4) was labeled with Cy3 using a commercially available kit (FluoroLink Cy3 Reactive Dye; Amersham Pharmacia Biotech, Piscataway, NJ). Single-cell suspensions from spleen and lymph nodes were prepared and processed for flow cytometry on a FACStar or FACScan (Becton Dickinson, Mountain View, CA) as described previously (18). B cells (surface Ig (sIg)-expressing cells) were removed by magnetic depletion using goat anti-mouse IgG-coated Dynabeads (Dynal, Hamburg, Germany) (4–6 beads/cell).

Cloning and expression of rART2.2

Cloning and transfection of plasmid pME.CD8LF-ART2.2 for expressing N-terminally FLAG-tagged ART2.2 in mammalian cells were as described previously (18). The structural gene-encoding mouse ART2.2 represents one member of a tandem duplication and is denoted as *Art2b*.

Stimulation of cells

Cells were washed and resuspended in RPMI 1640 medium (Life Technologies, Rockville, MD) (1–5 $\times$ 10^6/ml) at 37°C. PMA (Sigma, Deisenhofen, Germany) and IC-3 (provided by R. Black; Immunex) were added as indicated, and cells were incubated at 37°C for the indicated times. For kinetic analyses, cells were prestained with appropriately labeled Abs before addition of PMA. AI4 splenocytes from NOD.AI4$\alpha\beta$Tg mice were dispensed into round-bottom wells containing 10–15 islets freshly prepared from NOD.*scid* RIPB7 mice. Anti-CD3 Abs were coated onto 96-well microtiter plates (10 mg/ml) by incubation overnight at 4°C. Plates were washed twice with PBS before adding cells (2 $\times$ 10^6/ml) suspended in RPMI 1640 medium. Plates were briefly centrifuged (2 min, 500 $\times$ *g*) to settle cells onto the bottom of the wells. Cells were incubated for 2 h at 37°C. Cells were subjected to immunofluorescence analyses as described above.

Treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC)

Cell suspensions were washed twice with PBS, resuspended at 10^8 cells/ml in RPMI 1640 medium with 1 U PI-PLC (Molecular Probes, Eugene, OR), and incubated on a roller for 1 h at 37°C. Cells were pelleted by centrifugation, washed in PBS, and processed for FACS analysis.

Immunoprecipitation, Western blot, and enzyme analyses

Cell supernatants were cleared by high-speed centrifugation and subjected to immunoprecipitation and Western blot analyses as described previously (18, 23). Immunoprecipitates were resuspended in 50 μl enzyme buffer (20 mM Tris, pH 8.0, 1 mM ADP-ribose, 1 mM DTT, 1 μCi ^{32}P-NAD$^+$ with or without 1 mM agmatine) and were incubated for 1 h at 37°C. Matrix-bound protein was pelleted by centrifugation, and supernatants were analyzed by TLC as described previously (24). For immunodepletion of ART2.2, supernatants of activated cells (100 μl) were incubated twice with mAb immobilized on protein G Sepharose beads (Amersham Pharmacia Biotech) (2 μg mAb/20 μl matrix) for 60 min at 4°C. Supernatants were supplemented with 1 mM ADP-ribose, 1 mM DTT, 1 mM agmatine, and 10 μM ^{32}P-NAD$^+$ (1 μCi), incubated for 1 h at 37°C, cleared of protein with Strataclean resin (Stratagene, Hilden, Germany), and analyzed by TLC as above.

Treatment of cells with etheno-NAD

Single-cell suspensions were resuspended at 5 $\times$ 10^7 cells/ml in RPMI 1640 medium with 100 μM etheno-NAD (Sigma) and incubated on a roller for 30 min at 37°C. Cells were pelleted by centrifugation, washed four times in ice-cold PBS, and processed for FACS analysis by indirect staining with etheno-adenosine-specific mAb 1G4 (mouse IgG1) (22) and FITC-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany).

Results

ART2.2 is released from Ag-primed cytotoxic T cells upon TCR cross-linking or activation of protein kinase C

We had previously shown that ART2.2 is down-modulated from the cell surface of mouse splenic and lymph node T cells upon treatment with the phosphokinase C activator PMA (18). We wondered whether ART2.2 would also be down-modulated from Ag-primed cells. To address this question we used a model system in which ART2.2$^+$ splenocytes harboring the AI4 transgenic TCR (19) were primed by their cognate Ag, an as yet unidentified pancreatic β cell Ag. Splenocytes obtained from AI4$\alpha\beta$Tg mice were plated onto pancreatic islets obtained from NOD.*scid* RIPB7 transgenic mice. After 7 days of culture, cells were harvested and restimulated either by plating onto anti-CD3-coated plates or by treatment with PMA. The results shown in Fig. 1 demonstrate that cell surface expression of ART2.2, indeed, is lost on >60% of cells within 2 h after plating on anti-CD3-coated plates (*c*) and on >95% of cells upon treatment with PMA (*b*). Presumably, the small fraction of cells on CD3-coated plates (*c*) that retain ART2.2 expression correspond to cells that did not contact the anti-CD3-coated surface of the culture well.

Loss of ART2.2 from the cell surface of PMA-activated T cells is blocked by the metalloprotease inhibitor IC-3

When we analyzed other GPI-anchored proteins (Thy-1 and Ly6C), we noticed that they—in contrast to ART2.2—did not disappear from the cell surface upon T cell activation (18). This indicated that loss of ART2.2 from the surface of activated T cells was not caused by a general activation of a GPI anchor-cleaving enzyme, such as PI-PLC, that would release ART2.2 from its GPI anchor. In contrast, we noticed that the disappearance of ART2.2 from the cell surface of activated T cells resembled the disappearance CD62L, a type I membrane protein. Because CD62L has recently been shown to be shed from activated T cells by the PMA-activated

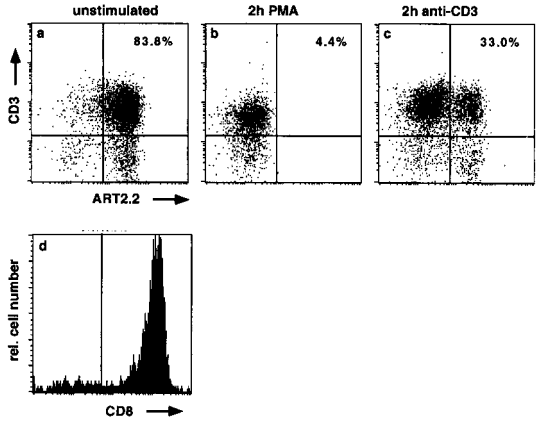

FIGURE 1. Down-modulation of ART2.2 cell surface expression upon restimulation of Ag-primed AI4 CTLs. AI4 splenocytes were cultivated for 7 days in the presence of their cognate Ag (NOD.RIP-B7/SzLt mouse islets). Cells were harvested and transferred to uncoated (*a*, *b*, and *d*) or anti-CD3-coated (*c*) plates and incubated for 2 h at 37°C in the presence (*b*) or absence (*other panels*) of PMA. Cells were then stained with PE-conjugated anti-CD8 (*d*) or with FITC-conjugated anti-ART2.2 and PE-conjugated anti-CD3 (*a–c*). Numbers indicate the percentage of ART2.2$^+$ cells within the subpopulation of CD3$^+$ cells.

membrane-anchored multidomain metalloprotease TACE (5), we suspected that ART2.2 might also be cleaved by TACE or another metalloprotease. To test this hypothesis, we analyzed whether PMA-mediated down-modulation of ART2.2 from lymph node T cells could be inhibited by the metalloprotease inhibitor IC-3 (25).

The results shown in Fig. 2 demonstrate that ART2.2, like CD62L, disappears from the cell surface of $CD4^+$ and $CD8^+$ lymph node cells within 120 min of incubation in the presence of PMA (Fig. 2, *b* and *e* vs *a* and *d*). Furthermore, PMA-induced loss of cell surface expression is almost completely inhibited by IC-3 (Fig 2, *c* and *f*). These results strongly suggest that ART2.2, like CD62L, is shed from activated T cells by a IC-3-sensitive metalloprotease.

Loss of ART2.2 from the cell surface of PMA-activated T cells resembles the loss of CD62L in dose response and kinetics

If ART2.2 and CD62L were released from activated T cells by a similar activation-induced pathway, their disappearance from the cell surface should exhibit similar kinetics and dose responses to PMA. The results shown in Fig. 3, indeed, demonstrate nearly identical PMA dose responses of ART2.2 and CD62L release from NOD splenic T cells (Ki_{50} 0.05 ng/ml). Moreover, ART2.2 and CD62L show very similar kinetics of disappearance from the cell surface in the presence of PMA (50% release within 15 min) (Fig. 4).

ART2.2 immunoprecipitated from the supernatant of PMA-activated T cells exhibits a slightly lower M_r *than ART2.2 released upon cleavage of the GPI anchor with PI-PLC*

If ART2.2 were released from activated T cells by proteolytic processing, we reasoned that ART2.2 should be recoverable from the supernatant of activated cells and, depending on the site of cleavage, should exhibit faster migration in SDS-PAGE analyses compared with native ART2.2. Indeed, using Nika102, ART2.2 could be immunoprecipitated from the supernatants of PMA-activated T cells. For comparison, we immunoprecipitated ART2.2 from the supernatants of T cells that had been treated with bacterial PI-PLC, which cleaves ART2.2 from the cell surface at its GPI anchor (18). The results shown in Fig. 5 demonstrate that ART2.2 released from PMA-activated lymph node cells migrates slightly faster than does ART2.2 released by PI-PLC from these cells (*lane 2* vs *lane 4*). These results indicate that ART2.2, like CD62L (26), is cleaved close to its membrane anchor upon PMA-mediated T cell activation.

We also examined whether mouse EL4 lymphoma cells stably transfected with *Art2b* cDNA were capable of expressing cell surface ART2.2 and shedding it upon activation with PMA. In these transfectants, the 8-aa FLAG epitope tag DYKDDDDK, followed

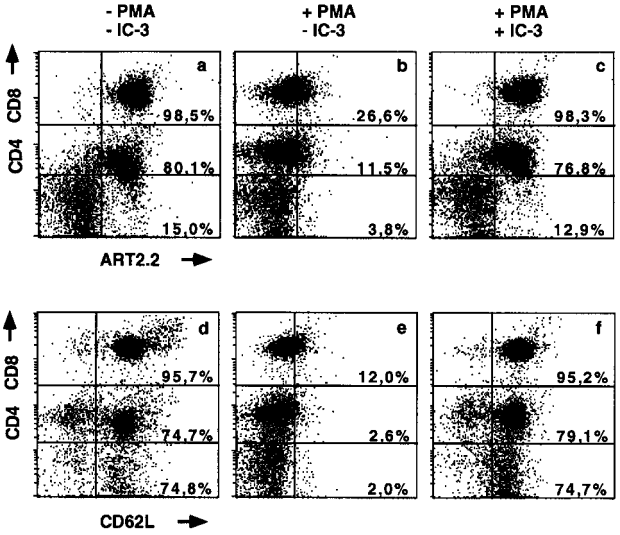

FIGURE 2. IC-3 blocks release of ART2.2 (*A*) and CD62L (*B*) from the cell surface of PMA-activated lymphocytes. Lymph node cells obtained from an 8-wk-old C57BL/6J mouse were stained with FITC-conjugated anti-ART2.2 (*a–c*) or anti-CD62L (*d–f*) and a combination of PE-conjugated anti-CD8 and Cy3-conjugated anti-CD4 before addition of PMA. Cells were incubated for 2 h at 37°C in normal medium (*a* and *d*) or in medium containing 100 ng/ml PMA with (*c* and *f*) or without (*b* and *e*) 200 μM IC-3. Numbers indicate the percentage of $ART2.2^+$ cells (*a–c*) or $CD62L^+$ cells (*d–f*) within subpopulations of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ cells.

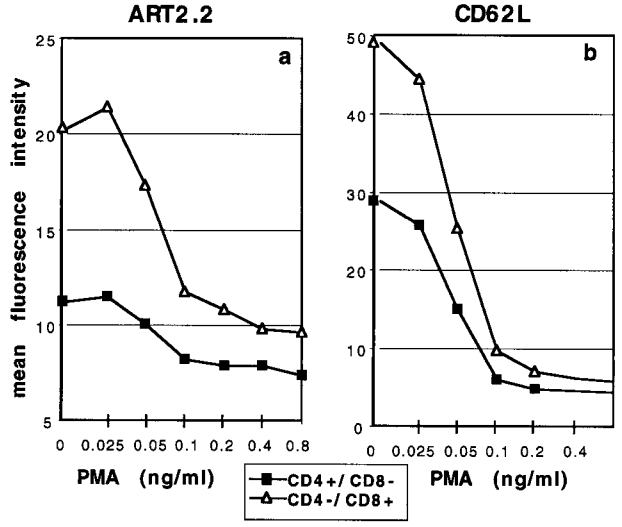

FIGURE 3. Dose response of ART2.2 (*a*) and CD62L (*b*) release after PMA-mediated cell activation. Splenocytes obtained from 6-wk-old NOD/Lt mice were stained for expression of ART2.2, CD62L, CD8, and CD4 as in Fig. 2 before addition of PMA. Cells were incubated for 2 h at 37°C with the indicated concentrations of PMA and then analyzed by flow cytometry.

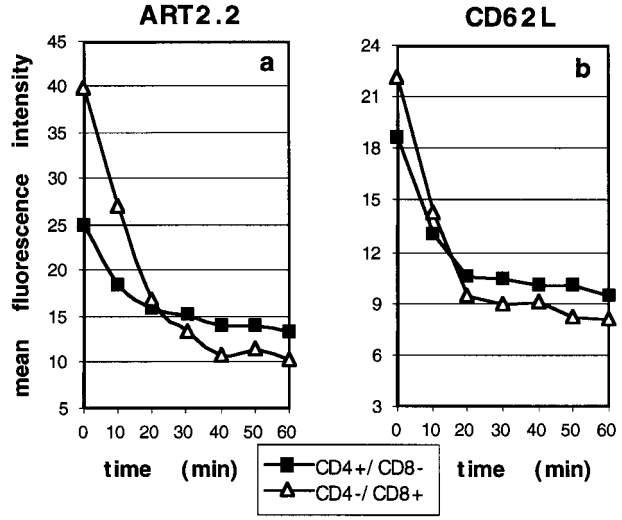

FIGURE 4. Kinetics of ART2.2 (*a*) and CD62L (*b*) release after PMA-mediated cell activation. Splenocytes obtained from 6-wk-old NOR/Lt mice were stained for expression of ART2.2, CD62L, CD8, and CD4 as in Fig. 2 before addition of PMA (100 ng/ml). Cells were placed in a 37°C water bath and aliquots of cells were subjected to flow cytometric analysis at the indicated time points.

by a 2-aa linker (GR) derived from the *Bgl*II-cloning site, is engineered onto the N terminus of ART2.2. The results shown in Fig. 5 (*lanes 6–8*) show that PMA activation of *Art2b*-transfected EL4 lymphoma cells, indeed, leads to release of ART2.2 into the cell supernatant. As in the case of lymph node cells, ART2.2 shed from EL4 cells upon PMA activation exhibits a slightly reduced M_r compared with ART2.2 released from these cells with PI-PLC (*lane 6* vs *lane 8*). Moreover, epitope-tagged rART2.2 exhibits a slightly higher M_r than does native ART2.2 from lymph node cells (compare *lanes 6* and *8* vs *lanes 2* and *4*).

ART2.2 shed from activated T cells is enzymatically active

Metalloprotease-mediated shedding of T cell surface proteins has been proposed to represent a mechanism for releasing soluble intercellular regulators (3, 27). ART2.2 belongs to the family of arginine-specific ARTs, which modify target proteins by transferring the ADP-ribose unit from NAD^+ to arginine residues in target proteins (7). If ART2.2 were released from activated T cells as an intercellular regulator, we reasoned that it should be enzymatically active.

We have previously shown that a soluble form of rART2.2 ADP-ribosylates a number of targets in vitro, including a tag-specific Ab when bound to epitope-tagged ART2.2, arginine-rich histones, and the arginine analogue agmatine (23). When we tested the enzyme activity of ART2.2 bound to Nika102 and Nika109, another mAb derived from the same fusion, we noticed a significant inhibition of ART2.2 enzyme activity when bound to mAb Nika102 but not when bound to mAb Nika109 (data not shown). Moreover, both light and heavy chains of Nika109, when bound to rART2.2, served as efficient substrates for ART2.2-mediated ADP-ribosylation.

To assess whether native ART2.2 upon release from T cells is enzymatically active and, thus, a potential intercellular regulator, we immunoprecipitated ART2.2 from the supernatants of PMA-activated or PI-PLC-treated lymph node cells and incubated the ART2.2 immunoprecipitates with radio-labeled NAD^+ in the presence or absence of agmatine. The results shown in Fig. 6 demonstrate that ART2.2 shed from the surface of PMA-activated T cells (*lanes 2* and *6*) catalyzes ADP-ribosylation of agmatine as efficiently as ART2.2 released by PI-PLC (*lanes 4* and *8*). Moreover, both forms of soluble ART2.2 catalyze ADP-ribosylation of histones as well as the immunoprecipitating Ab with similar efficiencies (data not shown). These results demonstrate that native ART2.2, as purified from the supernatants of PI-PLC-treated and PMA-activated T cells, exhibits potent ART activity, as has been previously observed for rART2.2 (23).

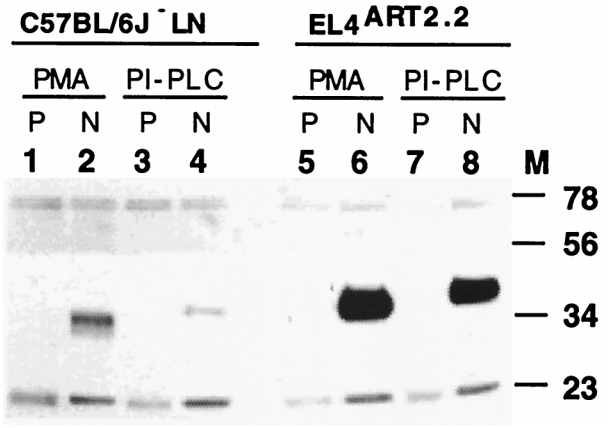

FIGURE 5. ART2.2 recovered from the supernatant of PMA-activated T cells by immunoprecipitation exhibits a lower M_r than ART2.2 released upon cleavage of the GPI anchor with PI-PLC. Immunoprecipitates were prepared from supernatants of C57BL/6J lymph node cells (*lanes 1–4*) and ART2.2-transfected EL4 lymphoma cells (*lanes 5–8*) after treatment of cells with PMA (*lanes 1, 2, 5,* and *6*) or PI-PLC (*lanes 3, 4, 7,* and *8*). Immunoprecipitates were prepared with protein G-Sepharose and rat preimmune serum (*lanes 1, 3, 5,* and *7*) or ART2.2-specific mAb Nika102 (*lanes 2, 4, 6,* and *8*) and subjected to SDS-PAGE and Western blot analyses. Blots were stained with the enhanced chemiluminescence system using rabbit anti-ART2.2 peptide antiserum K48 and peroxidase-conjugated goat anti-rabbit IgG. The common band at 24 kDa corresponds to the Ab light chain.

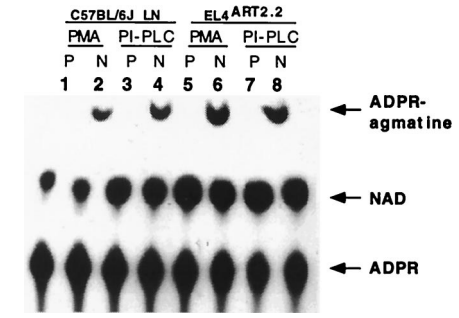

FIGURE 6. Shed ART2.2 recovered from the supernatant of activated T cells by immunoprecipitation is enzymatically active. ART2.2 immunoprecipitates were prepared from supernatants of C57BL/6J lymph node cells and ART2.2-transfected EL4 cells as in Fig. 5, except that mAb Nika109 was used instead of Nika102. Immunoprecipitates were incubated with ^{32}P-NAD^+ in the presence of agmatine (1 mM) and ADP-ribose (1 mM) for 1 h at 37°C. Aliquots of the reaction products were analyzed by TLC. Chromatograms were subjected to autoradiography by exposure to X-OMAT AR film (Kodak, Rochester, NY) for 16 h at room temperature.

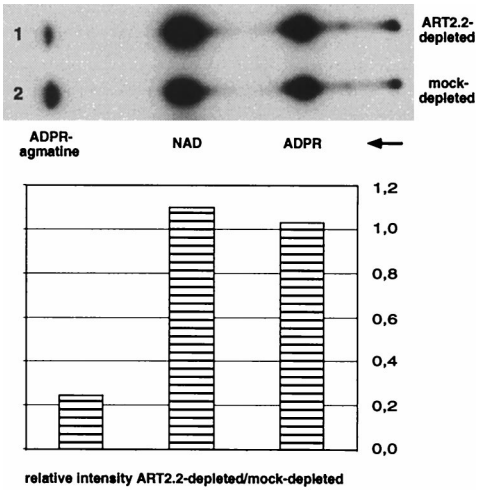

FIGURE 7. Removal of ART2.2 from the supernatant of activated T cells by immunoprecipitation depletes most of the agmatine-ADP-ribosylating activity. Supernatants of C57BL/6J lymph node T cells were subjected to two successive immunoprecipitation steps with immobilized mAb Nika102 (*lane 1*) or isotype-matched (IgG2a) control mAb B4 (*lane 2*). Clarified supernatants were incubated with ^{32}P-NAD^+ in the presence of agmatine (1 mM) and ADP-ribose (2 mM) for 1 h at 37°C. Aliquots of the reaction products were analyzed by TLC. Chromatograms were subjected to autoradiography by exposure to X-OMAT AR film (Kodak) for 16 h at room temperature (*top*). The arrow indicates the direction of migration. Individual spots were analyzed by densitometry using the NIH Image software. The relative intensities of the respective spots in *lanes 1* and *2* are presented graphically (*bottom*).

ART2.2 is the main ART activity in the supernatant of PMA-activated T cells from C57BL/6J mice

In the mouse, five other cDNAs encoding ART2.2-related ectoenzymes (designated ART1–5) have been cloned to date (8). Evidence for the expression of ART1 and ART5 on lymphoma cells has been reported (28, 29). Because mAbs against other ARTs are still lacking, we could not directly address the question of whether metalloprotease-mediated shedding is a property unique to ART2.2 or a more general property of GPI-anchored ARTs. To assess whether ART2.2 is the main enzyme activity released by activated lymph node T cells, we examined whether ART activity could be removed from the supernatants of these cells by immunoprecipitation with Nika102. For control, we subjected an identical aliquot of the supernatant to immunoprecipitation with an isotype-matched control Ab. The results shown in Fig. 7 demonstrate that immunoprecipitation with Nika102 removes most, but not all, agmatine ADP-ribosylating enzyme activity from the supernatants of activated T cells.

T cell activation dramatically reduces the sensitivity of T cell surface proteins to ADP-ribosylation

It has previously been shown that treatment of lymph node cells with NAD^+ leads to ADP-ribosylation of cell surface proteins and suppresses important T cell functions (11, 13–15). Treatment of T cells with PI-PLC, which removes GPI-anchored cell surface proteins including cell surface ART activity, rendered cells resistant to the immunosuppressive effects of NAD^+. These observations suggested to us that shedding of ART2.2 by activated T cells might change the responsiveness of T cell surface proteins to ADP-ribosylation. To test this hypothesis, we analyzed the capacity of resting and PMA-activated T cells to ADP-ribosylate cell surface proteins (Fig. 8, *A* and *B*).

To assess the responsiveness of cell surface proteins to ADP-ribosylation, we exploited the ability of ART2.2 to use etheno-NAD as substrate, resulting in etheno-ADP-ribosylation of target proteins. We established a FACS assay for detecting etheno-ADP-ribosylated cell surface proteins using the etheno-adenosine-specific mAb 1G4 (22) (Fig. 8). Incubation of ART2.2-transfected EL4 lymphoma cells with etheno-NAD (100 μM) for 30 min at 37°C resulted in staining with the 1G4 mAb, while ART2.2-negative parental EL4 cells were completely resistant to labeling with the 1G4 Ab (data not shown). Similarly, incubation of purified lymph node cells with etheno-NAD for 30 min at 37°C resulted in 1G4 staining of most $CD4^+$ and $CD8^+$ cells, but not of cells expressing sIg (Fig. 8*A*, *panel 2* vs *panel 1*). Activation of total lymph node cells (data not shown) or purified sIg-negative lymph node cells (Fig. 8*B*, *panel 2*) for 2 h with PMA dramatically reduced the capacity of T cells to incorporate etheno-ADPR.

A recent report on CD38 knockout mice showed that this cell surface Ag accounts for most of the ecto-NAD-glycohydrolase activity on mouse lymphocytes (30, 31). We were, thus, concerned that the presence of the potent NAD-glycohydrolase activity of CD38 might influence substrate availability for ART2.2-mediated ADP-ribosylation. Therefore, we also monitored the cell surface expression of CD38 in these experiments (Fig. 8*C*). Interestingly, expression of CD38 and ART2.2 seems to be inversely correlated, i.e., $sIg^+/ART2.2^-$ cells strongly express CD38, whereas $sIg^-/ART2.2^+$ cells expose little if any CD38. Moreover, in contrast to ART2.2 (Fig. 8*C*, *panels 1* and *3*) and CD62L (Fig. 2, *D* and *E*), cell surface expression of CD38 is not altered significantly within 2 h after treatment with PMA (Fig. 8*C*, *panels 2* and *4*). Note also that labeling intensity of T cells with mAb 1G4 was reduced only slightly in the presence vs absence of $CD38^+/sIg^+$ cells (compare Fig. 8*A*, *panel 2* and Fig. 8*B*, *panel 1*). These results show that the

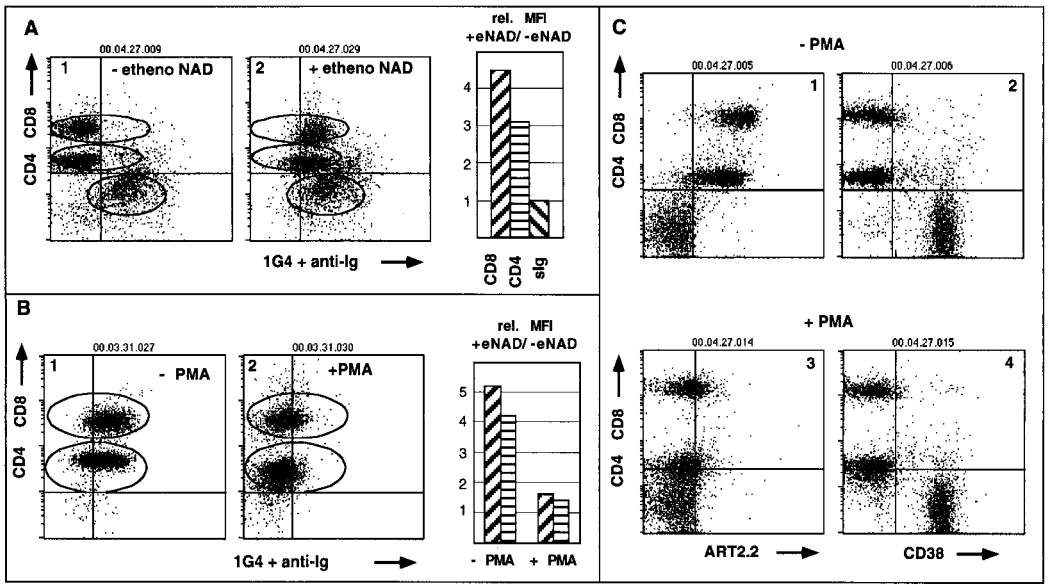

FIGURE 8. Loss of T cell surface ADP-ribosylation activity upon cell activation with PMA. *A*, C57BL/6J lymph node cells were incubated for 30 min without (*panel 1*) or with (*panel 2*) 100 μM etheno-NAD. Cells were then washed and stained with mAb 1G4 followed by FITC-conjugated anti-mouse IgG and a combination of PE-conjugated anti-CD8 and Cy3-conjugated anti-CD4. *B*, C57BL/6J lymph node cells were depleted of sIg-expressing cells by magnetic cell sorting. sIg-depleted cells were incubated for 2 h in the absence (*panel 1*) or presence (*panel 2*) of 100 ng/ml PMA, washed and stained for FACS analyses as in *A*. The mean fluorescence intensities of cells in the gated regions in *A* and *B* were calculated with the CellQuest software (Becton Dickinson). The results are represented graphically for the individual subpopulations as the relative mean *x*-axis fluorescence intensity of cells incubated with (+) vs without (−) etheno-NAD. *C*, C57BL/6J lymph node cells were incubated for 2 h in the absence (*panels 1* and *2*) or presence (*panels 3* and *4*) of 100 ng/ml PMA. Cells were washed and then stained with a combination of PE-conjugated anti-CD8 and Cy3-conjugated anti-CD4 and FITC-conjugated anti-ART2.2 (*panels 1* and *3*) or FITC-conjugated anti-CD38 (*panels 2* and *4*). Note that the staining of CD4 is reduced in PMA-activated cells due to endocytosis of CD4 (40), while that of ART2.2 is reduced due to shedding of ART2.2.

capacity to ADP-ribosylate cell surface proteins is positively correlated with the expression of ART2.2 but negatively correlated with the expression of CD38. Moreover, shedding of ART2.2 upon cell activation correlates with a markedly reduced capacity of T cells to ADP-ribosylate cell surface proteins.

Discussion

The results presented here show that T cell activation triggers shedding of the T cell ecto-ART, ART2.2 (Figs. 1, 2, and 5). Dose response, kinetics of release, and sensitivity of shedding of ART2.2 to the inhibitor IC-3 strongly suggest that shedding of ART2.2 is mediated either by the same enzyme that releases CD62L, i.e., TACE, or by a closely related metalloprotease (Figs. 2, 3, and 4). Shed ART2.2 is enzymatically active and ADP-ribosylates a number of substrates in vitro (Fig. 6). The sensitivity of T cells to ADP-ribosylation of cell surface proteins is dramatically reduced upon cell activation (Fig. 8). ART2.2 evidently accounts for most, if not all, of the ART activity in the supernatants of activated T cells from C57BL/6J mice (Fig. 7), a mouse strain known to express comparatively high amounts of ART2.2 but little if any ART2.1 (21, 32). However, because mAbs against other ARTs are still lacking, future studies will need to address whether metalloprotease-mediated shedding is a property unique to ART2.2 or a more general property of ecto-ARTs.

ART2.2 shed from PMA-activated T cells shows a slightly smaller M_r in SDS-PAGE than does ART2.2 released by cleavage of the GPI anchor, indicating that ART2.2 shedding from activated cells is caused by proteolytic cleavage close to the C terminus of ART2.2, i.e., close to its GPI anchor attachment site (Fig. 5). TACE has been shown to use an Ala-Val cleavage site in TNF-α (33). ART2.2 contains a similar motif, Ala^{275}-Val^{276}, 8 aa residues upstream of the predicted GPI anchor attachment site (21). Cleavage at this position would be compatible with the shift in m.w. that is observed when comparing PMA-released ART2.2 with PI-PLC-cleaved ART2.2 (Fig. 5). However, because the exact position of the GPI anchor attachment site in ART2.2 has not been yet determined and because motifs other than Ala-Val are compatible with metalloprotease cleavage (34), further studies will be required to identify the position of the metalloprotease cleavage site in ART2.2.

It is not unlikely that the modulation of cell surface ART2.2 observed upon T cell activation in vitro (Figs. 1, 2) reflects comparable cell surface ART2.2 modulations operating also during physiological immune responses in vivo. The finding that splenocytes with an activated phenotype, i.e., cells expressing the IL-2R, CD25, are $ART2.2^-$ (18) suggests that ART2.2, indeed, is modulated also during T cell activation in vivo.

It has been hypothesized that ART2.2 plays an immunoregulatory role on the basis of the observations that ART2.2 is capable of posttranslationally modifying other proteins, that expression of ART2.2 is restricted to T cells, and that its expression is developmentally regulated (7, 18). The results presented here lend support to this hypothesis and raise two interesting possibilities regarding ART2.2-mediated immune regulation: 1) shedding and reexpression of ART2.2 may serve to regulate the responsiveness of the T cell itself, e.g., rendering the cells insensitive and responsive, respectively, to the immunosuppressive effects of cell surface ADP-ribosylation reactions; and 2) ART2.2 may act as a secreted intercellular regulator that modulates the function of other cells or secretory proteins by ADP-ribosylation.

Our findings agree with previous studies showing the release of soluble ART activity upon stimulation of allogeneic mouse CTLs (16) and chicken heterophils (17). Removal of ART activity from the cell surface of allogeneic mouse CTLs rendered these cells refractory to the regulatory effects of cell surface ADP-ribosylation, e.g., to inhibition of signal transduction and target cell binding (11, 35). In the case of chicken heterophils, it was shown that the released enzyme could ADP-ribosylate the extracellular signaling peptide, tuftsin (17), and, furthermore, that ADP-ribosylation of tuftsin blocks its capacity to activate the phagocytic activity of macrophages. Because ART-specific Abs were lacking, the molecular identity of the ART activities was not clarified in these previous studies.

With respect to the possible role of ART2.2 as a secreted intercellular regulator, it may be of interest to point out that the distantly related toxin bacterial ARTs translocate from the extracellular environment through the cell membrane of target cells to ADP-ribosylate targets in the cytoplasm (36, 37). The possibility that translocation to the cytoplasm of target cells is a mode of action also for ART2.2 shed from activated T cells warrants attention.

The hypothesis that cell surface expression of ART2.2 is an indicator of the responsiveness of a T cell to immune regulation and/or of its potential to act as a regulatory cell is also consistent with a number of studies that showed a correlation among cell surface expression of RT6, the rat homologue of ART2.2, and the regulatory potential of T cells in the BB rat model of insulin-dependent diabetes mellitus: activated, $RT6^-$ cells adoptively transfer disease, while $RT6^+$ cells mediate protection (38, 39). Our results suggest that $ART2.2^-$ cells are less responsive to be down-regulated by ADP-ribosylation of cell surface proteins, thereby turning these cells into potentially more aggressive effector cells. Moreover, $ART2.2^+$ but not $ART2.2^-$ cells could serve as a reservoir for an immune regulator to be released via metalloprotease-mediated shedding, thereby rendering $ART2.2^+$ cells more competent regulatory cells.

We conclude that ART2.2 is one of the potential intercellular regulators released by activated T cells via metalloprotease-mediated cleavage from the cell surface. Furthermore, shedding of ART2.2 alters the responsiveness of T cells to ART2.2-mediated ADP-ribosylation of cell surface proteins.

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